DEMONSTRATION OF A METABOLIC GRID AT AN EARLY STEP IN THE STREPTONIGRIN BIOSYNTHETIC PATHWAY IN *STREPTOMYCES FLOCCULUS*

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The enzyme activities which catalyze the conversion of tryptophan to β-methyltryptophan by two different routes have been demonstrated in cell-free extracts of streptonigrin-producing *Streptomyces flocculus*. The first route involves direct methylation of tryptophan by a *C*-methyltransferase. The second involves transamination of tryptophan to indolepyruvate, methylation of indolepyruvate to β-methylindolepyruvate, followed by a reverse transamination reaction to yield β-methyltryptophan. The direct methylation route was confirmed by the fact that the methyltransferase activity is still present after the transaminase has been inactivated by hydroxylamine treatment. The L-tryptophan *C*-methyltransferase has been purified 30-fold by ammonium sulfate precipitation and a Sephadex G-150 column. The indolepyruvate *C*-methyltransferase activity copurified with the tryptophan *C*-methyltransferase activity, but the transaminase did not. These results show that a metabolic grid exists for the first antibiotic-committed step of the streptonigrin biosynthetic pathway.

Streptonigrin is an antitumor antibiotic produced by *Streptomyces flocculus* ATCC 13257. Although the complete biosynthetic pathway to streptonigrin is not known, GOULD and DARLING[1] have shown that β-methyltryptophan is produced by the organism and that both tryptophan and β-methyltryptophan are incorporated into the phenylpicolinic acid portion of the streptonigrin molecule. We have previously demonstrated that the dialyzed cell-free extracts of *S. flocculus*, in the absence of added cofactors, can catalyze the transfer of the methyl group of *S*-adenosylmethionine to tryptophan to form β-methyltryptophan (HARTLEY and SPEEDIE, in press). Since the β-position of the tryptophan side chain is not obviously activated for nucleophilic attack upon the methyl group of *S*-adenosylmethionine, we hypothesized that either the *C*-methyltransferase activates the β-position of the side chain in order that direct methylation of tryptophan can occur, or that the formation of β-methyltryptophan involves a three step process, *i.e.*, transamination of tryptophan to indolepyruvate, followed by methylation at the β-position (which can now be activated by keto-enol tautomerism), followed by a reverse transamination step to convert β-methylindolepyruvate to β-methyltryptophan. The pathways are illustrated in Fig. 1. SPEEDIE *et al.*[2] have previously reported two analogous enzymes, a tryptophan transaminase and an indolepyruvate *C*-methyltransferase, in the biosynthetic pathway to the antibiotic indolmycin in *Streptomyces griseus*. However, direct methylation of tryptophan was not catalyzed by the cell-free extracts of *S. griseus*.

In this paper we present evidence that in *S. flocculus* the enzyme activities for both the direct methylation of tryptophan and the transaminase-methyltransferase route to β-methyltryptophan are present in the cell-free extract, thus suggesting that a metabolic grid is operating at the first antibiotic-committed step of the streptonigrin biosynthetic pathway.

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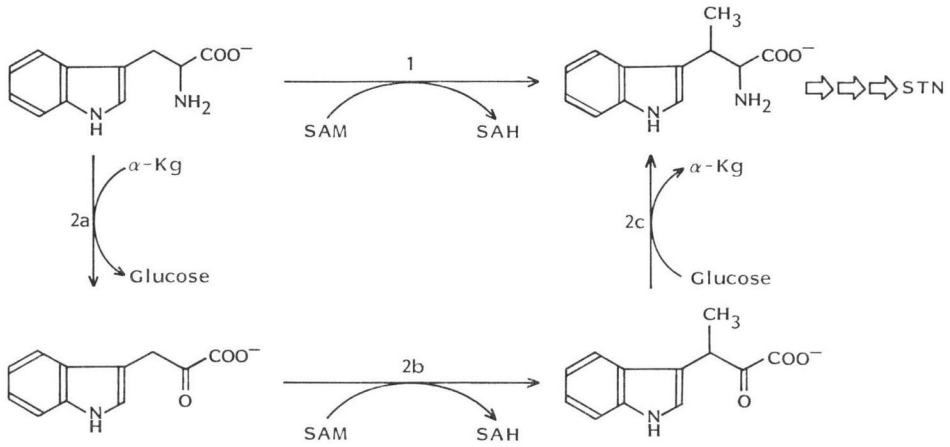
Fig. 1. Postulated metabolic grid for conversion of tryptophan to β-methyltryptophan in *S. flocculus*. 1=Tryptophan 3-methyltransferase; 2a=tryptophan aminotransferase; 2b=indolepyruvate 3-methyltransferase; 2c=3-methylindolepyruvate aminotransferase. (SAM=*S*-Adenosylmethionine; SAH=*S*-adenosylhomocysteine; α-Kg=α-ketoglutarate; STN=streptonigrin).



Materials and Methods

Materials

Yeast extract, beef extract, and Emerson agar were purchased from Difco (Detroit, Mich.). Trypticase soy broth, dextrose, and Gelysate peptone were from Baltimore Biological Laboratories (Baltimore, MD). *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (~55 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Its radiochemical purity was examined periodically by paper chromatography. DL-[*methylene*-^{14}C]Tryptophan (specific activity 57 mCi/mmol) was from Amersham (Arlington Heights, IL). All other chemicals were reagent grade or better and available commercially.

Culture Conditions

Streptomyces flocculus (ATCC 13257) was maintained on Emerson agar slants. After inoculation, the slants were incubated at 26~27°C for 5 to 7 days and then stored at 4°C until used. Inoculum for shake cultures was prepared by washing the surface of a slant with 5 ml of sterile water and gently scraping the surface with a sterile pipet. The liquid was suction-filtered through sterile Whatman No. 1 filter paper in a Buchner funnel. The resulting spore suspension (3 to 6×10^6 spores/ml) was added to 100 ml of Emerson medium (beef extract 4.0 g, Gelysate peptone 4.0 g, yeast extract 1.0 g, sodium chloride 2.5 g, dextrose 10.0 g and distilled water to 1 liter) in a 1-liter Erlenmeyer flask and incubated at 26~27°C on a New Brunswick Scientific rotary shaker at 150 rpm for 48 hours. Twenty-five ml of this inoculum were added to 500 ml of Emerson medium in a 4-liter Erlenmeyer flask and incubated as above for the appropriate length of time.

Cell Extracts and Enzyme Preparation

Fifty-four to 62-hour cultures of *S. flocculus* were harvested by suction filtration. After washing the mycelial pad once with distilled water, it was resuspended in 0.01 M sodium phosphate buffer, pH 8.0, with 1 mM dithiothreitol (buffer I) to form a thick slurry (1 g cells/3 ml buffer). All subsequent steps were carried out at 4°C. A cell-free extract was prepared by sonic disruption of the cell slurry for 5 minutes at 23 W with an ultrasonic disintegrator (Branson Sonifier, Model W-140, 1 cm probe). Cellular debris was removed by centrifugation at $30,000\times g$ for 20 minutes (Damon/IEC Division, IEC B-20A Centrifuge). The resulting supernatant, dialyzed overnight against 2 changes of 2 liters of buffer I without dithiothreitol (buffer II), was used as the crude enzyme preparation.

Enzyme Assays

S-Adenosylmethionine: L-Tryptophan 3-methyltransferase was assayed by incubating 1.0 ml of en-

zyme preparation with 5 mM each L-tryptophan and *S*-adenosyl-L-methionine, 0.03 mM pyridoxal 5-phosphate and 0.1 μCi *S*-adenosyl-L-[*methyl*-^{14}C]methionine in a total volume of 1.4 ml. The control consisted of 1 ml of enzyme which had been boiled for 5 minutes and the same concentration of all other reagents. After incubation at 37°C for 90 minutes the reaction was stopped by the addition of 2 ml of water-saturated butanol. The assay tubes were agitated on a vortex mixer for 15~30 seconds and centrifuged at low speed to separate the two phases. The butanol was removed and the extraction was repeated twice more with 1.5 ml of butanol each time. This procedure removed 70~80% of the β-methyltryptophan from the aqueous phase. The butanol extracts were pooled and an aliquot was dried under nitrogen. The dried residue was redissolved in methanol and chromatographed on silica gel plates (EM Laboratories aluminum-backed plates or J. T. Baker glass-backed plates, both 0.25 mm thickness, with fluorescent indicator) in 2-butanone - pyridine - water - acetic acid (70: 15: 15: 2) or on cellulose plates (EM Laboratories aluminum-backed plates, 0.25 mm thickness with fluorescent indicator) in butanol - acetic acid - water (12: 3: 5). After development, the plates were air dried and sprayed with a solution of 0.25% ninhydrin in 2-propanol to detect tryptophan and β-methyltryptophan. The zone corresponding to β-methyltryptophan was scraped into a mini-scintillation vial (Research Products, Inc., Mount Prospect, IL) containing 0.40 ml of methanol. After agitation, 6 ml of scintillation cocktail (Beckman Ready-Solv EP) were added. The samples were counted with a liquid scintillation counter (Searle Mark III, Model 6880) to at least 2% standard error. Radiochromatogram scanning was used to detect zones of radioactivity on TLC plates. When indolepyruvate was used as a substrate for the *C*-methyltransferase it was added in place of L-tryptophan to a final concentration of 5 mM and incubated as before. Other reagent concentrations and reaction conditions were the same as when tryptophan was the substrate. To extract β-methylindolepyruvate, the β-methylated product that would be expected if the enzyme could use indolepyruvate as a substrate, the reaction mixture was acidified to pH 3~4 with 1 N tartaric acid and extracted with one volume of butyl acetate[2]. The butyl acetate was spotted directly on silica gel plates and developed in ethyl acetate - 2-propanol - acetic acid (5: 4: 1). The zones corresponding to standard β-methylindolepyruvate, as detected by UV quenching (254 nm) or by a purple color after spraying with VAN URK's reagent, were scraped into miniscintillation vials containing 0.40 ml methanol. After agitation, 6 ml scintillation cocktail were added and the radioactivity was determined by scintillation counting.

Tryptophan: 2-Oxoglutarate aminotransferase (EC 2.6.1.27) was assayed in a Beckman DU-8 UV-Visible spectrophotometer using the assay of SPEEDIE and coworkers[2] with the following modifications: L-Tryptophan and α-ketoglutarate were 5 mM, the pH of the assay was 8.0 and the temperature was 37°C. A UV scan of the reaction mixture was performed after 10 minutes, using enzyme preparation and L-tryptophan in the reference cuvette. In addition, the formation of indolepyruvic acid from tryptophan was confirmed by incubating 1 ml of enzyme preparation with 5 mM α-ketoglutarate, 0.03 mM pyridoxal 5-phosphate, 5 mM L-tryptophan and D,L-[*methylene*-^{14}C]tryptophan in a final volume of 1.4 ml at 37°C for 2 hours. The reaction was stopped by extraction with 2 volumes of ethyl acetate. The ethyl acetate was concentrated *in vacuo* and chromatographed on silica gel plates. Following development in ethyl acetate - 2-propanol - acetic acid (5: 4: 1), the plates were scanned with a Packard Model 7201 Radiochromatogram scanner, and sprayed with VAN URK's reagent[3].

To Detect Glutamate: β-Methylindolepyruvate aminotransferase, it was first necessary to enzymatically prepare β-methylindolepyruvate. β-Methyltryptophan, isomer A (1 mg), was incubated with 4 mg (1.0 U) of L-amino acid oxidase, 2 mg (10^4 U) catalase and 2 drops of 1-octanol in 1 ml of buffer I at 30°C for 2 hours, during which time oxygen was bubbled through the reaction mixture. The reaction was stopped by acidification with 1 N tartaric acid to pH 3 and the reaction mixture was extracted twice with 2 volumes of ethyl acetate. The combined extract was concentrated to dryness *in vacuo*, redissolved in a small amount of ethyl acetate and spotted on silica gel plates and developed in ethyl acetate - 2-propanol - acetic acid (5: 4: 1). One edge of the plate was sprayed with VAN URK's reagent to identify the β-methylindolepyruvate zone. The corresponding zone was scraped from the remainder of the plate, and divided in half. One portion was extracted with 3×3 ml ethyl acetate, which was subsequently taken to dryness *in vacuo*. The dried extract was incubated for 1 hour at 37°C with 1 ml crude enzyme, 5 mM L-glutamate, and 0.03 mM pyridoxal 5-phosphate. A parallel reaction was run using the other half of the β-methyl-

indolepyruvate still bound to silica gel in place of the dried extract since the stability of β-methylindolepyruvate to elution and evaporation was uncertain. A control consisted of all the reaction components except β-methylindolepyruvate. The reactions were stopped by extraction with 3×1.5 ml of water saturated butanol. The butanol extracts were dried and an aliquot chromatographed as described for the work-up of β-methyltryptophan from the methyltransferase assay. The remaining portion of the dried butanol extract was further purified by extraction with 2×1 ml chloroform to remove lipid contaminants and then injected onto HPLC using a previously described system (HARTLEY and SPEEDIE, in press) and the peak corresponding to authentic β-methyltryptophan was quantitated.

C-Methyltransferase Purification

Solid ammonium sulfate was added slowly with stirring to the crude enzyme preparation to 35% saturation. After stirring for 20 minutes, the precipitate was removed by centrifugation at 30,000×*g* for 20 minutes. The resulting supernatant was brought to 60% saturation with solid ammonium sulfate as described above. The precipitate was collected by centrifugation as before and either frozen at −20°C for storage or resuspended in buffer I to a final protein concentration of 6~8 mg/ml. This represented the 35~60% ammonium sulfate fraction. Before assaying, the fraction was dialyzed against 2 changes of 2 liters of buffer II.

The resuspended ammonium sulfate fraction was applied to a Sephadex G-150 column (2.5 cm× 33.5 cm) which had been equilibrated with buffer I. Elution was effected with the same buffer and 5 ml fractions were collected. The fractions with the *C*-methyltransferase activity were pooled and the protein was precipitated by adding solid ammonium sulfate to a final concentration of 45 g/100 ml. After centrifugation as before, the precipitate was redissolved in buffer I to a final concentration of 0.2 mg/ml and dialyzed overnight against 2 changes of 2 liters of buffer II.

Protein was determined by the biuret method[4] for crude and ammonium sulfate fractions and by the Biorad method for Sephadex G-150 fractions. Bovine serum albumin (Fraction V) was used as the standard.

Apoenzyme Formation

The procedure of MARTIN *et al.*[5] was used to form apoenzymes. The ammonium sulfate precipitated enzyme from the Sephadex G-150 column was redissolved in 0.05 M imidazoleacetic acid buffer, pH 7.2, and dialyzed overnight. Hydroxylamine was added to the enzyme preparation to a final concentration of 0.2 M. This solution was dialyzed for 60 minutes at room temperature against 0.05 M imidazole - acetic acid buffer, pH 7.2, containing 1 mM dithiothreitol and 1 mM hydroxylamine. The protein solution was then exhaustively dialyzed at 4°C, against buffer I. The control (untreated) enzyme was subjected to the same conditions without exposure to hydroxylamine.

Results

C-Methyltransferases

As shown in Table 1, crude, dialyzed, cell-free extracts of *S. flocculus* were able to catalyze the transfer of a methyl group from *S*-adenosylmethionine to the β-position of the side chains of both L-tryptophan and indolepyruvic acid. D-Tryptophan was not a substrate. When the rates of methylation of the two substrates were compared (under reaction conditions optimized for the tryptophan *C*-methyltransferase), the specific activity of the indolepyruvate methyltransferase is 1.3-fold that of the tryptophan methyltransferase reaction. It should be noted that the rate for each substrate was calculated with correction for the differing extraction efficiencies for the two products.

Aminotransferases

Tryptophan aminotransferase activity, as measured by increased absorbance at 305 nm due to indolepyruvate, was observed when the dialyzed enzyme preparation was incubated with L-tryptophan, pyridoxal 5-phosphate and α-ketoglutarate. As seen in Table 2, no increase in absorbance was observed

unless α-ketoglutarate was added to the assay. Although addition of pyridoxal 5-phosphate increased the reaction rate, its addition was not required for the reaction with the enzyme preparation which had been dialyzed 2 hours. When pyruvate was used as the amino acceptor, the rate of formation indolepyruvate was only 60~70% the rate observed with α-ketoglutarate.

Table 1. Substrates for *C*-methyltransferase reaction.

Substrate	Product (μmol)[a]	
	β-Methyltryptophan	β-Methylindolepyruvate
L-Tryptophan	0.691[b]	0
D-Tryptophan	0	0
Indolepyruvic acid	0.027[b]	0.968

[a] Reaction consisted of 5 mM substrate, 5 mM *S*-adenosyl-L-methionine, 6.4 mg 35~65% ammonium sulfate fraction protein, 10 mM sodium phosphate, pH 8.0, in a total volume of 1.4 ml. The reaction was run for 90 minutes at 37°C. The quantity of product is a total for 1.4 ml reaction mixture after 90 minutes.

[b] Corrected for extraction efficiency of product.

Table 2. Cofactors for tryptophan aminotransferase activity.

Reaction mixture	Specific activity (mU/mg)
Complete[a]	0.036
Complete — pyridoxal phosphate	0.019
Complete — α-ketoglutarate	0
Boiled enzyme (complete)	0

[a] "Complete" reaction mixture was comprised of 0.98 mg protein from the 35~60% ammonium sulfate precipitant fraction, 5 mM L-tryptophan, 5 mM α-ketoglutarate, 0.03 mM pyridoxal 5-phosphate, in 1.4 ml of 10 mM sodium phosphate buffer, pH 8.0.

When the reverse reaction, *i.e.* transamination of indolepyruvate to tryptophan, was monitored by decreased absorbance at 305 nm, a decrease in absorbance was observed whether enzyme or an amino donor was present or not (Table 3), suggesting non-enzymatic loss of indolepyruvate at a rate substantially greater than any enzyme-mediated conversion. This could mean that the reverse aminotransferase activity was not present in the enzyme preparation, or that the amino donors screened, including glutamate, glutamine and alanine, were not appro-

Table 3. Assay for the transamination of indolepyruvate to tryptophan.

Assay conditions	ΔA_{305nm}/minute	Percent of complete
Complete[a]	−0.0256	100
Complete — pyridoxal phosphate	−0.0091	35.5
Complete — amino donor	−0.0248	96.9
Complete — enzyme	−0.0250	97.6

[a] 1 ml enzyme+5 mM indolepyruvate+0.03 mM pyridoxal 5-phosphate+5 mM L-alanine, L-glutamate or L-glutamine. The enzyme was from crude fraction preparation which had been dialyzed for at least two hours prior to the assay.

Table 4. Typical purification scheme of tryptophan (TMT) and indolepyruvate (IMT) *C*-methyltransferase activities.

Enzyme preparation	Total enzyme (mg)	Specific activity (mU/mg)[a]	Purification factor	Total activity (mU)	Fraction of initial total units (%)	Ratio of activities (TMT/IMT)[b]
Crude						
TMT	225	0.73	—	163	100	
IMT	〃	0.97	—	217	100	0.75
Ammonium sulfate						
TMT	57.9	0.90	1.24	52.3	31.7	0.72
IMT	〃	1.26	1.31	73.0	33.7	
Sephadex G-150						
TMT	1.14	17.8	24.5	20.0	12.4	0.72
IMT	〃	24.3	25.2	27.7	12.8	

[a] Data from reaction mixture corrected for difference in extraction efficiency of products.

[b] Tryptophan *C*-methyltransferase/indolepyruvate *C*-methyltransferase.

Table 5. Typical purification scheme of aminotransferase (AT) and *C*-methyltransferase (CMT) activities.

Enzyme preparation	Total enzyme (mg)	Specific activity (mU/mg)[a]	Purification factor	Total activity (mU)	Fraction of initial total units (%)	Ratio of activities (CMT/AT)
Crude						
CMT	567.0	0.17	—	97	100	4.41
AT	〃	0.038	—	22	100	
Ammonium sulfate						
CMT	216	0.29	1.70	63	64.6	11.6
AT	〃	0.025	—	5.4	24.5	
Sephadex G-150						
CMT	11.9	4.6	26.5	55	56.1	688
AT	〃	0.007	—	0.08	0.36	

[a] CMT uncorrected for extraction efficiency of β-methytryptophan.

Table 6. Apoenzyme formation by hydroxylamine treatment.

Enzyme activity	Hydroxylamine-treated	Addition of pyridoxal phosphate (0.03 mM)	Percent activity[a]
C-Methyltransferase	−	+	100
C-Methyltransferase	+	−	72.4
C-Methyltransferase	+	+	82.0
Aminotransferase	−	+	100
Aminotransferase	+	−	0
Aminotransferase	+	+	95.5

[a] Percent activity compared to untreated control with pyridoxal phosphate added.

priate for transamination in this direction. In any case, the increase in absorbance at 305 nm, observed when tryptophan and α-ketoglutarate are substrates, is a minimum measure of indolepyruvate formation, since indolepyruvate undergoes some type of breakdown under the assay conditions.

When β-methylindolepyruvate, made by incubating β-methyltryptophan with L-amino acid oxidase, was incubated with the crude enzyme fraction and glutamate, a ninhydrin-positive spot which cochromatographed with β-methyltryptophan was observed. The presence of β-methyltryptophan was confirmed by HPLC of the dried, extracted reaction mixture. Quantitation by HPLC showed 25.6 μg of β-methyltryptophan in the final extract. This would represent 2.1×10^{-4} U of enzyme per ml of cell-free extract.

Enzyme Purification

The *C*-methyltransferase activity was purified 1.3 to 1.7-fold by ammonium sulfate precipitation and an additional 12 to 15-fold by chromatography on Sephadex G-150. The results of a typical purification are shown in Table 4. Further attempts at purification were unsuccessful.

Since the aminotransferase activity was still present in the combined fractions containing the majority of *C*-methyltransferase activity after the Sephadex G-150 step, we suspected that the two activities might exist in an enzyme complex. However, the results in Table 5 suggest that this is not the case, since the ratios of *C*-methyltransferase/aminotransferase activities are not constant at each step of purification. Purification of the *C*-methyltransferase appears to eliminate most of the aminotransferase activity.

On the other hand, when the enzyme at each stage of purification was also assayed for indolepyruvate methyltransferase activity, the ratio of β-methyltryptophan to β-methylindolepyruvate was constant throughout the purification procedure (Table 4). The two methyltransferase activities appear to be due either to one enzyme with flexible substrate specificity or to a tight complex of two enzymes.

Apoenzyme Formation

When the enzyme preparation from the Sephadex G-150 column was treated with the hydroxyl-

amine procedure no aminotransferase activity was present unless pyridoxal 5-phosphate was added (Table 6). However, more than 70% of the *C*-methyltransferase activity remained, allowing the *C*-methyltransferase to be assayed in the absence of aminotransferase activity. These results indicate that transamination of tryptophan is not required for methylation.

Discussion

A metabolic grid is a series of parallel pathways which begin and end with the same compounds, but in which the reactions occur in different sequences. Metabolic grids are involved in the biosynthetic pathways of tylosin[6] and erythromycin[7] although not at the first committed step. The possibility of a metabolic grid in the biosynthetic pathway of streptonigrin was first suggested when we isolated from *S. flocculus* an aminotransferase and a *C*-methyltransferase, both of which utilize tryptophan as substrate. The original question of sufficient activation of tryptophan for methylation *via* nucleophilic attack of the β-carbon on the methyl group of *S*-adenosylmethionine could be approached by visualizing a transamination step followed by a methylation step. This has been seen in indolmycin biosynthesis by *S. griseus* where tryptophan must first be transaminated to indolepyruvate before methylation can occur[2]. In this case, transamination is obligatory and no metabolic grid exists at that point in the biosynthesis of indolmycin. However, evidence for direct methylation of tryptophan by the *C*-methyltransferase suggested that perhaps two pathways could operate in this organism. There are two pieces of evidence suggesting direct methylation of tryptophan: 1) product resulted even when no α-ketoglutarate was added to the *C*-methyltransferase assays and the aminotransferase activity is absolutely dependent on the addition of α-ketoglutarate, and 2) after apoenzyme formation by hydroxylamine treatment, no aminotransferase activity was present unless pyridoxal 5-phosphate is added and yet β-methyltryptophan formation continued even in the absence of pyridoxal 5-phosphate (albeit, at a slower rate with the hydroxylamine-treated preparation), thus demonstrating that direct methylation must occur as well. If the metabolic grid exists and both aminotransferase steps, *i.e.* tryptophan to indolepyruvate and β-methylindolepyruvate to β-methyltryptophan, are occurring at the same time, it is conceivable that no additional substrate would be required since autocatalysis could generate sufficient cofactor for both reactions. In fact, when indolepyruvate was incubated with the ammonium sulfate or Sephadex G-150 fractions in the *C*-methyltransferase assay, with no added amino donors or cofactors, small amounts of β-methyltryptophan as well as β-methylindolepyruvate were detected among the reaction products (Table 1), implying that enough charged pyridoxamine is present to catalyze a small amount of transamination. However, the hydroxylamine-treated preparation, which had no detectable aminotransferase activity, catalyzed the formation of β-methyltryptophan, thus confirming the direct methylation route.

The establishment of the tryptophan to indolepyruvate and β-methylindolepyruvate to β-methyltryptophan transamination steps confirms that in *S. flocculus* two pathways exist which could form β-methyltryptophan. Whether or not both pathways to β-methyltryptophan formation operate at the same rate, or one is preferred, is not known, but could be elucidated by determining the K_m and V_{max} values for purified aminotransferase and *C*-methyltransferase. This would enable selection of the rate-limiting step in the grid and allow definition of the more prominent pathway. Preliminary indications of substrate specificity of the *C*-methyltransferase activity (Table 1) suggest that indolepyruvate may be a slightly better substrate for the *C*-methyltransferase than tryptophan. Direct competition experiments would be useful in this regard. However, if transamination is much slower than methylation, as suggested by the specific activities of the two enzymes, the net result may be that direct methylation of tryptophan to β-methyltryptophan is the preferred biosynthetic route.

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